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Antibodies to distinct epitopes on the CD40 molecule co-operate in stimulation and can be used for the detection of soluble CD40

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SUMMARY

The B-cell surface protein, CD40, belongs to the tumour necrosis factor/nerve growth factor (TNF/NGF) receptor family and plays a crucial role in T cell-dependent B-cell activation. Ligation of this receptor with antibodies or its recently defined ligand, gp39, generates an intracellular signal that, when combined with triggering of surface immunoglobulin or the interleukin-4 (IL-4) receptor, induces a variety of stimulatory effects in B cells. In this study we provide further evidence for the importance of receptor cross-linking in generating this signal and we also report on the presence of a soluble form of CD40. A new CD40 monoclonal antibody (mAb), 17:40, was found to synergize with other CD40 antibodies (mAb89 and S2C6) in inducing proliferation as well as IgE synthesis in IL-4-treated tonsillar B cells. However, both this mAb and mAb89 failed to co-operate with a soluble construct of the CD40 ligand, whereas such co-operation was seen with the S2C6 antibody. Cross-inhibition experiments showed that the 17:40 mAb recognized an epitope that was clearly distinct from that seen by S2C6 and mAb89. Although directed to separate epitopes, both 17:40 and mAb89 completely blocked binding of gp39 to its receptor, while the S2C6 mAb only partially interfered with this binding. The findings suggest a close relationship between the degree of receptor clustering and the strength of the delivered signal. With the access to antibodies recognizing distinct structures on CD40 we also established a sandwich enzyme-linked immunosorbent assay for quantitative determinations of the antigen. With this assay we could demonstrate the presence of a soluble form of CD40 (sCD40) in culture supernatants. The fact that sCD40 also retained its ligand-binding capacity indicates that it may have an important regulatory role and modulate the T cell-dependent stimulation via CD40. Both the finding of soluble receptors and the need for receptor clustering are features that CD40 share with other members of the TNF/NGF receptor family.

INTRODUCTION

The CD40 cell surface receptor is expressed on immature and mature B lymphocytes, dendritic cells and some normal and malignant epithelial cells.[1–3] Costimulation of B cells with antibodies to CD40 has been shown to lead to a number of effects including the induction of short- and long-term proliferation,[4–7] cell enlargement,[8] homotypic adhesion[8–10] and protection against apoptosis.[11] Structurally, CD40 belongs to a family of receptors, which includes the low affinity nerve growth factor (NGF)-receptor, the two tumour necrosis factor (TNF)-receptors, the fas/APO antigen as well as the two less well characterized receptors CD27 and CD30.[3] The signal transduction pathways engaged by CD40 or by the other receptors of this family, are not well understood. However, receptor cross-linking appears to be one of the requirements[5] and specific phosphorylation of several intracellular proteins has also been observed in response to CD40 stimulation.[12]

Recently the CD40 ligand, gp39, was cloned and characterized as a transmembrane cell surface glycoprotein of 39 000 MW.[13,14] With an expression restricted to activated T cells, predominantly of the $CD4^+$ subtype, it supports the need for physical contact between B cells and T helper cells during T cell-dependent B-cell activation. Most of the stimulatory effects, previously observed with antibodies, have also been possible to corroborate with use of the ligand.[15] Furthermore, and demonstrating the physiological relevance of this receptor–ligand system, there have recently been several reports showing that mutational defects in gp39 in patients with X-linked hyper-IgM syndrome are the functional explanation for the inherent immunodeficiency of these individuals.[16–19] A similar structural relationship as that observed between the receptors of the NGF/TNF receptor family has also been noted for several of their ligands. Thus, gp39 is about 30% homologous with both TNF-α and TNF-β[14,20] and a similar degree of homology was recently described for the Fas-ligand.[21] Moreover, all of these ligands are thought to occur as multimers and both types of

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TNF as well as gp39 have been predicted to be expressed as biologically active trimers.[20]

In the present study we describe a new anti-CD40 monoclonal antibody (mAb), which is directed to an epitope that is distinct from that recognized by most other CD40 antibodies. The effect of this mAb in costimulation with other CD40 antibodies was investigated and a sandwich enzyme-linked immunosorbent assay (ELISA) established with the help of the antibody was used for the detection of a soluble form of CD40 in culture supernatants.

MATERIALS AND METHODS

Reagents

The phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) was obtained from Sigma Chemical Co. (St Louis, MO). Recombinant IL-4 was purchased from Genzyme (Boston, MA) and the anti-CD18 mAb, MHM23 (IgG1) was from Dakopatts a/s (Roskilde, Denmark). The two CD40 mAb, S2C6[1] and mAb89[7] (both IgG1) were produced in the laboratory and a kind gift from Dr H. Yssel (DNAX, Palo Alto), respectively. A soluble fusion protein composed of the extracellular part of CD40 fused with the Fc domain of human IgG (CD40–Ig),[22] as well as a fusion protein comprising the external part of the CD40 ligand, gp39, linked to mouse CD8[14] were obtained from COS cell transfectants.

Cells and preparation of cell lysates

Resting B lymphocytes were purified from human tonsils by negative selection and gradient centrifugation as described earlier.[23] The preparations contained >97% CD20-positive cells and ≤1% CD3 or CD11b-positive cells. Other cell types used were the Burkitt lymphoma cell, Raji, and the bladder carcinoma cell lines HU549, T24, J82 and 5637. The origin and culturing conditions for these cell lines have been given elsewhere.[24]

Quantitative analyses of CD40 were made by ELISA on soluble cell lysates and on culture supernatants. Cell lysates were prepared as described elsewhere (1). Briefly, harvested cells were solubilized on ice for 30 min in phosphate-buffered saline (PBS) containing 0·1% NP-40 and protease inhibitors (40×10^6 cells/ml). Insoluble material was removed by centrifugation. Culture supernatants containing soluble CD40 (sCD40) were collected from dense cell cultures of high viability (>95% trypan blue-excluding cells). Cells and debris were removed by centrifugation (800 *g*) and passage through a 0·22 μm sterile filter. In some cases supernatants were also ultracentrifuged (100 000 *g*) for 2 hr at 4°. For assessment of ligand-binding capacity, sCD40 was also used after further purification on a S2C6-coupled Sepharose column.[25]

Aggregation, proliferation and IgE synthesis

B cells were stimulated by antibodies together with TPA or IL-4 in HEPES-buffered RPMI 1640 supplemented with 10% fetal calf serum, penicillin (100 IU/ml), streptomycin (100 μg/ml), glutamine (2 mM) (all from Gibco, Paisley, UK) and 2-mercaptoethanol. Cells were cultured at 1×10^6 cells/ml in flat-bottomed 96-well plates (Linbro Chemical Co., New Haven, CT) in a final volume of 0·2 ml/well. All tests were set up in triplicate. Aggregation was registered by microscopy 3 to 4 days after initiation of culture. For proliferation, cells were stimulated for 4 days and pulsed with [^{3}H]thymidine (1 μCi/well, 18·0 Ci/mmol, Amersham International, Amersham, UK) during the last 18 hr. After harvesting the cells, [^{3}H]thymidine incorporation was measured in a scintillation counter. Determinations of IgE-production were made by ELISA as described previously[9] using supernatants from cultures stimulated for 10 days.

Derivation and purification of the anti-CD40 mAb 17:40

For the production of new mAb to CD40, mice were immunized with CD40 purified from Raji cells by affinity chromatography as described earlier.[25] Hybridomas were established by fusion of immune splenocytes with SP2/0 myeloma cells and screening was performed by ELISA against the purified antigen. Monoclonality was obtained by subcloning and CD40 reactivity was confirmed by testing against purified recombinant CD40–Ig.

An IgM antibody, 17:40, obtained by this procedure was partially purified from culture supernatants. For this purpose the hybridoma was grown in medium with low protein content, i.e. DMEM supplemented with 1% Ultroser (Gibco). Supernatants were collected and after removal of cells and debris by centrifugation and filtration (0·45 μm) they were concentrated over a filter with a molecular weight cut-off of 300 000 MW (YM 300, Diaflo, Amicon, MA). After concentration (approximately 50-fold), samples were diluted 10-fold with PBS and concentration was resumed. This procedure was repeated twice and resulted in IgM preparations that were more than 60% pure as estimated from their concentration of IgM, determined in an Ig-ELISA, in relation to total protein.

Epitope analysis

The epitopes recognized by the different antibodies and the CD40 ligand were analysed in ELISA or by radioimmunoassay. Ninety-six-well break-apart ELISA plates (High-Sorp, Nunc, Roskilde, Denmark) were coated overnight with CD40–Ig (5 μg/ml) in PBS. After saturation with 1% bovine serum albumin (BSA) for 4 hr and washing in PBS–Tween (0·05%) serial dilutions of the different antibodies were added. Immediately thereafter, ^{125}I-labelled antibodies (S2C6 or mAb89), non-labelled 17:40 mAb or the CD40 ligand (gp39–CD8) were added in criss-cross combinations. After 4 hr of incubation, plates were washed and binding of the radioiodinated mAb was registered in a γ-counter. Wells containing detecting 17:40 mAb or gp39–CD8 were further incubated with an alkaline phosphatase (ALP)-conjugated goat antibody specific for mouse IgM (Sigma) and a biotinylated anti-mouse CD8 antibody (Becton Dickinson, Mountain View, CA), respectively, followed by addition of streptavidin ALP. Finally, after incubation and washing, the substrate p-nitrophenyl phosphate was added and the plates were read at 405 nm in an ELISA spectrophotometer.

Development of a CD40 ELISA: analysis of soluble CD40 (sCD40) and its interaction with the CD40 ligand

For quantitative analysis of CD40 in cell lysates and soluble CD40 (sCD40) in culture supernatants, a sandwich ELISA was established. ELISA plates were coated with S2C6 or mAb89 (5 μg/ml) overnight in 0·05 M sodium carbonate buffer (pH 9·6) and after coated with 1% BSA as described above. Test samples were diluted in PBS–Tween and incubated for 4 hr at room

Table 1. Stimulation of tonsillar B cells with TPA and anti-CD40 antibodies

Stimuli	[^{3}H]thymidine incorporation	
	Exp. 1	Exp. 2
Medium	820 ± 26	1020 ± 128
TPA (2 ng/ml)	3330 ± 145	6220 ± 1113
TPA + 17:40		
10 μg/ml	32 670 ± 2753	54 820 ± 1850
2	21 370 ± 1232	36 720 ± 915
0·4	9310 ± 1318	18 360 ± 2112
TPA + S2C6		
10 μg/ml	29 550 ± 1437	48 360 ± 3636
2	18 220 ± 1653	29 880 ± 63
0·4	7640 ± 103	11 990 ± 713

temperature. After washing, mAb 17:40 (1 μg/ml) was added and plates were incubated overnight at room temperature. The plates were finally incubated for 4 hr with the IgM-specific ALP conjugate (Sigma) before being developed by the addition of substrate.

To test the ligand binding capacity of sCD40 it was affinity-purified from Raji cell supernatants on a Sepharose-column coupled with S2C6 mAb. After elution with 0·7% acetic acid and measurements of the sCD40 concentration by the CD40 ELISA the purified material was coated onto ELISA plates. After saturation with 1% BSA, CD40 ligand (gp39–CD8) was added and incubated overnight at 4°. After washing, biotinylated anti-mouse CD8 antibody (Becton Dickinson) was added and after further incubation and washing, streptavidin ALP (Mabtech, Stockholm, Sweden) was added. Finally, the plates were developed and scored as described above.

RESULTS

Characterization of the CD40 mAb 17:40

A new CD40 mAb, 17:40, was obtained from a mouse immunized with affininty-purified CD40. This IgM antibody was equally reactive as the two CD40 mAb, S2C6 and mAb89, when tested in ELISA against affinity isolated CD40 or purified recombinant protein (CD40–Ig). The immunostaining of a variety of cell types gave a very similar picture as with the other antibodies and was confined to B cells and some malignant epithelial cells (data not shown). However, unlike the other mAb it did not react with the Epstein–Barr virus-producing marmoset B cell line, B95–8, indicating the recognition of a more species-restricted epitope.

The 17:40 mAb also displayed the typical costimulatory capacity observed by other CD40 antibodies. Thus, stimulation

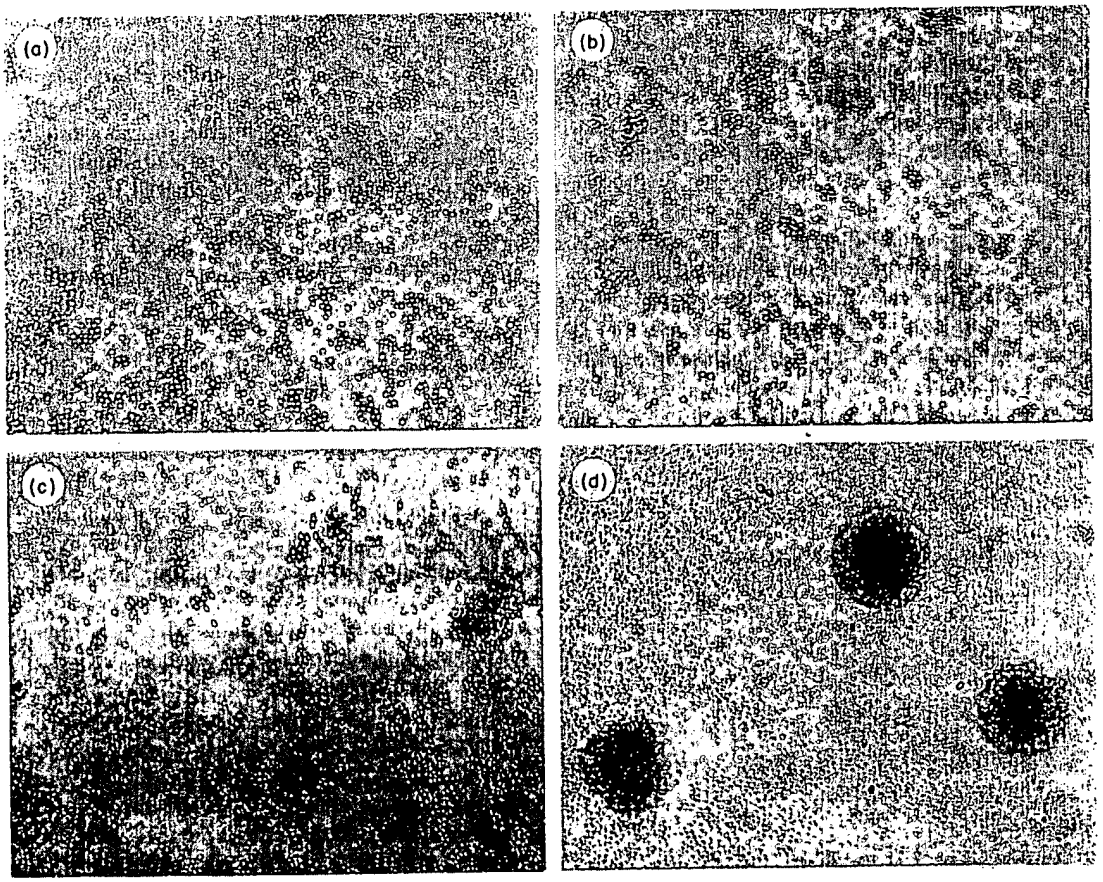

Figure 1. Homotypic aggregation of B cells cultured for 4 days with (a) medium; (b) IL-4 (500 iU/ml); (c) mAb 17:40 (1 μg/ml) and (d) IL-4 + 17:40. Magnification × 70.

Table 2. Effect of CD40 antibodies on IL-4-induced proliferation

Stimuli	[^{3}H]thymidine incorporation	
	Exp. 1	Exp. 2
Medium	250 ± 10	890 ± 460
IL-4 (500 iU/ml)	2020 ± 500	1230 ± 200
IL-4 + 17:40 1 μg/ml	7760 ± 580	7610 ± 150
2·5	8510 ± 130	7180 ± 760
IL-4 + S2C6 0·1	3230 ± 60	10340 ± 460
1·0	5170 ± 1	17730 ± 330
IL-4 + mAb89 0·1	14720 ± 690	26540 ± 400
1·0	20300 ± 1300	36740 ± 1890
IL-4 + gp39 500× dilution *	5480 ± 270	5970 ± 750
200×	10340 ± 1810	12710 ± 260
IL-4 + 17:40 1 + S2C6 0·1	69310 ± 4720	79590 ± 2730
IL-4 + 17:40 2·5 + S2C6 1·0	85900 ± 7520	85780 ± 3820
IL-4 + 17:40 1 + mAb89 0·1	53270 ± 2130	70120 ± 9720
IL-4 + 17:40 2·5 + mAb89 1·0	49800 ± 50	76720 ± 1
IL-4 + 17:40 1 + gp39 500×	9630 ± 1360	9190 ± 870
IL-4 + 17:40 2·5 + gp39 200×	11950 ± 1600	8610 ± 1300
IL-4 + S2C6 0·1 + mAb89 0·1	38410 ± 740	46520 ± 1610
IL-4 + S2C6 1·0 + mAb89 1·0	53140 ± 1910	66500 ± 2280
IL-4 + S2C6 0·1 + gp39 500×	28310 ± 1460	42090 ± 1110
IL-4 + S2C6 1·0 + gp39 200×	84400 ± 11900	76720 ± 8680
IL-4 + mAb89 0·1 + gp39 500×	12350 ± 790	17810 ± 100
IL-4 + mAb89 1·0 + gp39 200×	13620 ± 1650	16350 ± 250

*Diluted supernatant from COS cell transfectants producing the gp39-CD8 fusion protein.

of high density tonsillar B cells with the antibody together with TPA or IL-4 resulted in significant DNA-synthesis (Tables 1 and 2) and with IL-4 also in extensive cell clustering (Fig. 1). This homotypic aggregation was lymphocyte function-associated antigen (LFA)-1 dependent and could be partially prevented by the addition of antibodies to the β-chain (CD18) of LFA-1 (data not shown).

Co-operative stimulation by different CD40 antibodies and the CD40 ligand

In addition to testing the stimulatory capacity of the individual CD40 antibodies, they were also tried in combinations as well as together with a soluble construct of the CD40 ligand, gp39. As seen from Table 2 the 17:40 mAb was strongly synergistic with both the other mAb. This was most apparent for the S2C6 mAb where thymidine incorporation could be enhanced severalfold as compared with either mAb used alone. A co-operative response was also seen between S2C6 and mAb89 but this was markedly weaker and the effect was sometimes only additive.

When adding the antibodies together with gp39, S2C6 was the only mAb giving a costimulatory signal. The effect was clearly synergistic and similar to the combination of S2C6 and the 17:40 mAb. In contrast, simultaneous addition of ligand and mAb89 usually resulted in a suppressed DNA-synthesis as compared to stimulation with the antibody alone (Table 2). No combination of antibodies or ligand gave significant stimulation in the absence of IL-4 (data not shown).

Induction of IgE synthesis

In addition to the proliferative response, costimulation of B cells with CD40 antibodies and IL-4 has been shown to lead to IgE synthesis. To assess whether it was possible to enhance also this response by using combinations of antibodies, cells were stimulated for 10 days with IL-4 together with the antibodies in different combinations and IgE production was measured in the supernatants.

As seen in Table 3 the S2C6 and 17:40 mAb were both relatively poor inducers of IgE synthesis on their own. In contrast, cells stimulated with mAb89 and IL-4 produced significant levels of IgE. However, when combining the 17:40 and S2C6 mAb, a similar synergistic effect as that observed for proliferation was seen. While this finding was reproducible over a series of experiments the combination of mAb89 with 17:40

Table 3. Induction of IgE synthesis in tonsillar B cells stimulated with CD40 antibodies and IL-4

	IgE (ng/ml)	
Stimuli*	Exp. 1	Exp. 2
Medium	<0·03	<0·03
IL-4 (500 U/ml)	<0·03	<0·03
IL-4 + S2C6	1·85	<0·03
IL-4 + mAb89	9·70	3·70
IL-4 + 17:40	0·12	<0·03
IL-4 + S2C6 + mAb89	1·75	2·75
IL-4 + S2C6 + 17:40	5·10	7·70
IL-4 + mAb89 + 17:40	2·70	6·05

*Antibody concentrations were 0·1 μg/ml for mAb89 and S2C6 and 1 μg/ml for 17:40.

could result either in enhanced or decreased synthesis as compared to stimulation with mAb89 alone. This inconsistency appeared although proliferation of the same donors' lymphocytes in all cases showed increased proliferation in the presence of both antibodies.

Table 4. Cross-inhibition of anti-CD40 mAb and CD40 ligand binding

	% inhibition		
Inhibiting mAb (μg/ml)	17:40	S2C6	mAb89
[^{125}I]S2C6*			
10†	1·3	95·6	97·0
2	<1·0	76·4	71·9
0·4	<1·0	9·3	11·9
[^{125}I]mAb89*			
10	4·5	93·9	96·6
2	<1·0	77·6	78·2
0·4	<1·0	12·9	17·7
17:40‡			
10	ND	<1·0	<1·0
2	ND	9·8	5·4
0·4	ND	7·2	<1·0
gp39-CD8‡			
5	98·1	57·9	97·8
1	78·3	54·3	96·7
0·2	12·6	<1·0	22·4

*Amount of bound antibody (150 000 c.p.m. added) in the absence of inhibitory mAb was 97 100 c.p.m. for S2C6 and 114 800 c.p.m. for mAb89. Non-specific binding to wells without antigen was < 500 c.p.m.

† Concentration of inhibiting antibody.

‡ Results represent the inhibition of 17:40 or gp39-CD8 binding in ELISA. Binding was measured (OD 405 nm) after 20 min and was 0·974 (17:40) and 1·130 (gp39-CD8) in the absence of inhibitory mAb.

ND, not done.

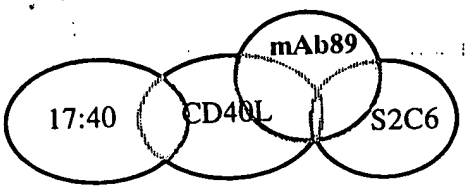

Figure 2. Suggested relationship between CD40 epitopes defined by the gp39 ligand and the antibodies 17:40, mAb89 and S2C6.

Epitope mapping of CD40 antibodies and the CD40 ligand

As receptor cross-linking is known to be a requirement for signalling via CD40, the costimulatory effects seen with certain antibody combinations might relate to their binding to different epitopes. Such binding to more than a single epitope could, in turn, result in a more extensive cross-linking and a stronger response signal. We therefore tested the epitope specificity of the different mAb. For this purpose ELISA plates were coated with CD40–Ig and the cross-blocking ability of the different mAb was measured. As shown in Table 4, mAb 17:40 did not affect binding of either of the two other antibodies. In contrast, S2C6 binding was clearly inhibitable by the addition of mAb89 and vice versa.

When investigating the ability of the antibodies to interfere with binding of the CD40-ligand all three mAb were to different extents able to do so (Table 4). The effect was greatest with 17:40 and mAb89, which both blocked ligand binding completely, while inhibition with mAb S2C6, even at high doses, never exceeded 60%.

Taken together, the results indicate that all three antibodies bind within the same region as the natural ligand. However, as outlined in Fig. 2 they define distinct epitopes that may be clearly overlapping as for mAb89 and the CD40 ligand, or not overlapping, as with the antibodies 17:40 and S2C6.

Development of a CD40 ELISA and the detection of soluble CD40 in culture supernatants

For quantitative determinations of CD40, a sandwich ELISA was established using the S2C6 mAb or mAb89 as catcher

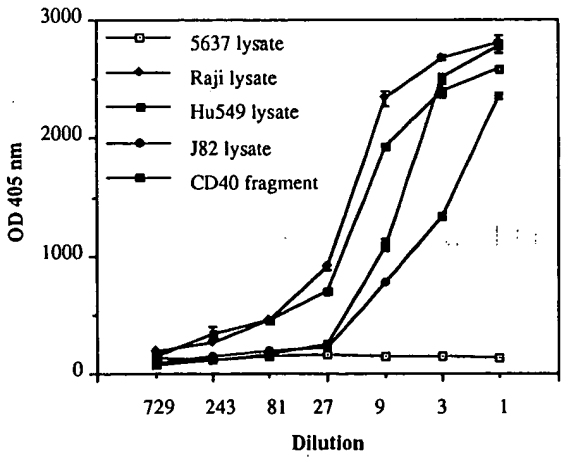

Figure 3. Detection of CD40 in lysates by ELISA. Samples were tested in duplicates and OD 405 nm was measured after 30 min. CD40–Ig was used as a standard.

Table 5. Binding of gp39 to sCD40 and CD40–Ig

Antigen	Coating concentration > (ng/ml)	OD 405 nm*
sCD40	30	0·580
	10	0·385
	3	0·115
	1	0·090
CD40–Ig	300	> 3·000
	100	2·530
	30	0·980

*OD was measured after 30 min. Background binding of biotinylated anti-CD8 to control wells (OD 0·118) has been subtracted from all values.

antibody and the 17:40 mAb for detection. This assay gave a sensitivity of approximately 0·5 ng/ml as determined from tests with purified soluble CD40–Ig as a standard. With this level of detection, CD40 was readily observed in cell lysates of CD40-positive cells (Raji, HU549, J82) but not in lysates of CD40-negative cells (5637) (Fig. 3).

Based on previous observations of soluble receptor molecules we also investigated whether the ELISA could be used for detection of soluble CD40 (sCD40). Supernatants from CD40-positive cell lines gave weak but significant signals in the ELISA. Although, the indicated levels were only slightly above the detection level of the assay (e.g. 2–5 ng/ml for the HU549 cell line and 10–15 ng/ml for Raji cells) they were reproducible and positive signals were never observed with supernatants from CD40-negative cells. The levels were also increased after concentration of supernatants over a 5000 MW cut-off filter. Moreover, and indicating the soluble nature of the detected material, the levels were not affected when supernatants were centrifuged for 2 hr at 100 000 *g*.

We further investigated whether the detected sCD40 also had retained its ability to bind the ligand. To do this sCD40 was first isolated from Raji cell supernatants by affinity chromatography and its concentration determined by the ELISA. Known concentrations of the purified material were then coated onto ELISA plates and its capacity to bind CD40 ligand was investigated. As shown in Table 5, significant binding was seen both to the purified sCD40 and to the CD40–Ig construct used as a positive control. Comparison between the two preparations indicate that a substantial proportion of the sCD40 retained its ligand binding activity.

DISCUSSION

Several of the previously reported antibodies to CD40 have been shown to react with the same or adjacent epitopes.[5,7] In the present study we describe a mAb, 17:40, which shares the stimulatory properties of other CD40 antibodies but is directed to a distinct epitope. This antibody acted in concert with other CD40 antibodies (mAb89 and S2C6) and could enhance IL-4/anti-CD40-induced DNA synthesis by up to fivefold as compared to stimulation with a single mAb. One of the antibodies, S2C6, also showed a strong co-operative response with the CD40 ligand. This was not the case with either mAb89 or the 17:40 antibody, which, in spite of recognizing separate epitopes, both efficiently blocked the interaction between CD40 and its ligand.

The observations are in line with and support previous indications of receptor cross-linking as an important requirement for signal transduction via CD40.[5] It further shows that the efficiency and degree of cross-linking may determine signalling strength and that the limited receptor clustering that can be achieved with a single mAb or the soluble ligand, is not sufficient to give optimal stimulation. In its natural state the CD40 ligand is thought to be expressed on the T-cell surface as a trimer.[20] As CD40 itself may also occur in multimeric form[26] these are all conditions that allow for extensive clustering of the receptor in the contact area between T and B cells. Thus, it is possible that the stronger stimulation that we observe here with combinations of antibodies better mimics the natural situation than does the use of a single mAb.

As shown in a recent study by Kwekkeboom *et al.*, not all antibodies to CD40 display stimulatory activity.[27] Three mAb raised by this group were unable to promote cell proliferation and could even abrogate the stimulation seen with other CD40 mAb. This indicates that antibodies, in addition to their cross-linking capacity, need to interact with a specific site on the molecule. It is therefore interesting that all the three mAb used in this study appear to bind within or close to the ligand binding site (Fig. 2). This is also true for the frequently used G28–5 mAb, which in cross-inhibition studies has been shown to block binding of both S2C6 and mAb89.[5,7]

Several recent studies have shown that stimulation of B cells with IL-4 and CD40 antibodies not only leads to proliferation but also to differentiation and IgE synthesis.[28–31] However, the reported amount of IgE produced in response to mAb89[29] has consistently been much higher than what we have been able to observe with the S2C6 mAb.[9] This relationship was also found here when having the opportunity to compare the two antibodies on the same cells. The 17:40 mAb, like S2C6, gave a low IgE production but when using combinations of the two antibodies, IgE production reached levels similar to those seen with mAb89. In search of an explanation to these differences in the IgE-inducing capacity of the different mAb we investigated the amount of IL-6 and soluble CD23 (sCD23) in stimulated cultures. Both of these factors are known to be released from CD40-stimulated cells[9,32] and they have also been claimed to be involved in the regulation of IgE synthesis.[28,33] Although some differences were observed, e.g. both IL-6 and sCD23 were found at slightly higher concentrations in cultures stimulated with mAb89 than with either of the two other mAb, there appeared to be no clear-cut correlation between the concentration of these factors and the amount of IgE measured in the cultures (data not shown).

The fact that the antibodies recognized distinct epitopes was also exploited for the development of a sandwich ELISA. With this assay we could easily quantify CD40 in crude cell lysates and, more important, we could also demonstrate the presence of a soluble form of CD40 in culture supernantants. This latter observation indicates that CD40 like the two structurally related TNF receptors[34–36] may be proteolytically cleaved and shedded from the cell surface. The fact that sCD40 displayed ligand-binding capacity indicates that it may have a functional role. Most easily conceivable is that it acts as an antagonist and inhibits the interaction between ligand and the intact cell

surface receptor. Such neutralization of ligand activity with soluble TNF-receptors has been demonstrated *in vitro*[37] and when administered in model systems *in vivo* they have been shown to inhibit TNF-induced inflammation and endotoxic shock.[37,38] However, low concentrations of soluble TNF receptors have been claimed to have the opposite effect and enhance TNF activity. This is believed to be due to a stabilization of the active trimeric form of TNF by the soluble receptors.[39] That this could well be the case also for the CD40 ligand is suggested by the observation that ligand expression on T cells could be significantly prolonged in the presence of a soluble CD40 construct.[40] Furthermore, the CD40 ligand is structurally closely related to TNF-α and -β and like those is believed to exist as a trimer.[20] Thus, it is likely that sCD40 takes active part in modulating T cell-dependent B-cell stimulation. One main site for such stimulation is in the paracortical, T-cell-rich area of the lymph node. Interestingly, a third important cell type in this area, the interdigitating dendritic cell (IDC), has been reported to express high amounts of CD40[41] and it is possible that sCD40 released from these cells serves to modulate the stimulation in a positive or negative way. Another site where T–B cell collaboration is thought to occur is in the lymph node follicles. Also here the follicular dendritic cells (FDC), which are functionally distinct from IDCs, have been shown to express CD40.[42] Although the functional role of CD40 in these two unrelated types of dendritic cells remains to be investigated it would be interesting to study if and upon what signals sCD40 may be released from these cells.

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